DUSP2 methylation is a candidate biomarker of outcome in head and neck cancer

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Background: Biomarkers predictive of response to chemoradiotherapy (CRT) regimens for locally advanced head and neck squamous cell carcinoma (LA-HNSCC) are urgently required to identify patients in whom this approach is likely to be effective. TP53 mutations and epidermal growth factor (EGFR) overexpression are common markers of disease. Dual-specificity-phosphatase-2 (DUSP2) has an essential role in cell proliferation, cancer and immune responses.

Methods: Aberrant DUSP2 methylation was investigated by pyrosequencing in 5 HNSCC cell lines, 112 LA-HNSCC tumours. EGFR was investigated by immunohistochemistry and TP53 was analysed by sequencing.

Results: We demonstrate methylation-dependent transcriptional silencing of DUSP2 in HNSCC cell lines. In LA-HNSCC patients, aberrant methylation in the DUSP2 CpG island was present in 51/112 cases (45.5%). LA-HNSCC cases with wild-type TP53, overexpression of EGFR and unmethylated DUSP2 had the worst overall survival ($P \leq 0.001$).

Conclusions: DUSP2 methylation, when combined with EGFR and TP53, is a candidate biomarker of clinical outcome in LA-HNSCC treated with CRT.

Keywords: Head and neck cancer; biomarkers; dual-specificity-phosphatase-2 (DUSP2); epigenetics

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Introduction

Despite improving clinical outcomes, optimal protocols for administration of combined modality therapy and biomarkers to inform the optimal use of such therapy have not been definitively identified in head and neck squamous cell carcinoma (HNSCC) (1). We have previously shown that mutations and polymorphisms in *TP53* and in *MDM2* predict response and survival in HNSCC patients treated with platinum-based chemoradiotherapy (CRT) (2-4).

The dual specificity phosphatases (*DUSPs*) comprise a large family of genes encoding enzymes which catalyse the dephosphorylation of serine, threonine and tyrosine residues in different types of mitogen-activated protein kinases within the MAPK TXY (Thr-Xaa-Tyr) motif (5). DUSP2 is an inducible nuclear phosphatase highly expressed in activated immune cells (6,7).

DUSP2 acts as a tumour suppressor at least in part via its physiological substrate ERK2 (8,9). Phosphorylation of ERK1/2, via Ras/Mek/ERK pathway, activates cell

proliferation and survival (10-13) in response to a wide range of stimuli, including radiation, hypoxia and chemotherapeutic agents (14).

Silencing of *DUSP2* leads to prolonged activation of the ERK pathway (15) that in turn has been associated with many aspects of tumour phenotype (16-18). Nonetheless *DUSP2* has been involved in the initiation and development of acute leukaemia (19) and in the pathogenesis of human solid cancers, where a loss of its expression has been associated with breast, colon, lung, ovary, kidney and prostate tumours in hypoxic conditions (8,15). The strong relationship between hypoxia and downregulation of DUSP2 affords a mechanistic link with angiogenesis and metastasis (20).

Overexpression of EGFR, leading to proliferation, angiogenesis and metastasis is frequently observed in HNSCC (21), but correlation with hypoxia is still debated (22,23). *DUSP2* affects the sensitivity of cancer cells to chemotherapy *in vitro* (15). Moreover, *DUSP2* is a transcriptional target of wild-type p53 (24) a determinant of treatment response in HNSCC (25).

In the present study, we have tested the hypothesis that *DUSP2* is transcriptionally silenced by methylation in HNSCC and in turn investigated the role of *DUSP2* as determinant of clinical outcome in locally advanced head and neck squamous cell carcinoma (LA-HNSCC) treated with platinum-based CRT.

Methods

Cell lines

Five human HN cancer cell lines (CAL27, CAL33, HEp-2, HNO41, HNO91) were used to evaluate DUSP2 expression and aberrant methylation in the CpG island. Cells were routinely cultured as previously described (26).

Gene expression analysis

Real-time qRT-PCR was performed on RNA extracted from cell lines using the RNeasy Mini Kit (Qiagen, Crawley, West Sussex, UK) according to standard procedures. *DUSP2* expression was assessed by TaqMan PCR assay Hs00358879_m1 using the ABI-Prism 7000 Sequence Detection System (Life Technologies, Carlsbad, California, US). Beta-2-microglobulin (Hs99999907_m1) was used as a control housekeeping gene.

Clinical cases and molecular analyses

Formalin fixed paraffin embedded (FFPE) specimens were obtained at diagnosis from 129 LA- HNSCC patients (stage III and IV) in Cuneo, from 1998 to 2013.

The distribution of gender, age, performance status (PS), tumor size (T), lymph nodes (N), histological grade (G), smoking habit and primary sites is shown in *Table 1*.

Smokers were defined according to Ang *et al.* [2010] (27) with a cut-off of 10 pack-year.

Mutational analysis of *TP53* and genotyping of the SNP Pro72Arg (rs1042522) were performed as previously described (2-4). *TP53* mutations were classified according to the IARC *TP53* mutation database (http://wwwp53.iarc.fr/), the Poeta *et al.* [2007] (28) study and the Evolutionary Action score of *TP53* (EAp53) (http://mammoth.bcm.tmc.edu/EAp53/) (29). EGFR was quantified in 72 patients by IHC as already described (30).

HPV was searched by DNA-PCR using specific primer pairs for type 16 in 129 patients' tissues; IHC staining for p16 (31) was performed in 118 patients' tissues. This work was carried out in accordance with the Declaration of Helsinki. The study and the informed consent for biological samples collection and research proposal, obtained from patients, were approved by the Ethical Committee of S. Croce & Carle Teaching Hospital in Cuneo (approval n. 198/13).

Methylation analysis

DNA samples were extracted from cell lines and FFPE tissues using standard protocols. In particular, genomic DNA was purified by proteinase K digestion of 10 m sticks cut from paraffin sections using xylene–phenol protocol, as previously described (3).

DUSP2 CpG island methylation was analyzed by pyrosequencing (Biotage, Uppsala, Sweden). Following PCR primers were designed to amplify a fragment of 134 bp, covering part of IVS I and exon 2: F 5'-GTAGATAGGAGTTTTGGAGT-3'; R5'-BIOT-CTCTTCCCCTCCTTACAAA-3'. 500 ng genomic DNA were amplified and analysed by QCpG Software (Qiagen) as already reported (32).

We used a universal commercial human DNA (CpGenome Universal Methylated DNA, Millipore Corporation, Billerica, MA, USA) as methylated (met) control (average methylation 98%), while DNA obtained

Table 1 Characteristics of LA-HNSCC patients (N=129) and tumours

Characteristic	Number of patients (%)	Median age [range] years
Gender		
Male (M)	108 (83.7)	59 [36–78]
Female (F)	21 (16.3)	60 [46–75]
Performance status		
PS0	59 (45.7)	
PS1	66 (51.2)	
PS2	4 (3.1)	
NA	0	
Tumour size		
T1/T2	44 (34.1)	
T3/T4	82 (63.6)	
NA	3 (2.3)	
Nodal status		
N0	9 (7.0)	
N1	16 (12.4)	
N2/N3	101 (78.3)	
NA	3 (2.3)	
Grade		
G1/G2	62 (48.1)	
G3	61 (47.3)	
NA	6 (4.7)	
Smoke		
Heavy smokers	103 (79.8)	
Non smokers	10 (7.8)	
NA	16 (12.4)	
Primary sites		
Oropharynx	36 (27.9)	
Hypopharynx	41 (31.8)	
Larynx	21 (16.3)	
Oral cavity	26 (20.2)	
Nasopharynx	2 (1.6)	
Other sites	3 (2.3)	

NA, not available; LA-HNSCC, locally advanced head and neck squamous cell carcinoma.

from a pool of 5 healthy head and neck epithelia was employed as unmethylated (unmet) control [mean value of *DUSP2* CpG island methylation was 4% (range, 3–7%)]. On the basis of this consideration we established a methylation cut-off of 10%.

Statistical analysis

Relationships between *DUSP2* gene methylation, clinical (gender, tumour site, PS, T, N, G, smoke and clinical response) and molecular (SNP rs1042522 and *TP53* sequence) characteristics were analysed by cross-tabulation.

OS analysis was based on the time from diagnosis to death or last contact in which the survivors were censored. PFS analysis was based on the time from diagnosis to first event (loco-regional recurrence or distant metastasis); patients without an event were censored at their last follow-up.

OS and PFS curves were calculated using the Kaplan-Meier method, where statistical significance of each variable was tested with log-rank test.

Univariate analysis was performed on each variable, then for *DUSP2*/EGFR (N=72) and *DUSP2/TP53* (N=101), and lastly in 70 DUSP2/EGFR/TP53 patients' combination.

Significant variables identified in the univariate analysis with $P \leq 0.05$ entered into a multivariate analysis performed on 70 DUSP2/EGFR/TP53 patients. Same analysis was repeated considering variables with $P<0.20$. The level of significance was $P<0.05$. Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs). Statistical analyses were performed using SPSS version 13 (SPSS, Chicago, IL, USA) program.

Results

Methylation-dependent transcriptional silencing of DUSP2 in HNSCC cell lines

Analysis of *DUSP2* expression by qRT-PCR showed that the mRNA was undetectable in HNO91 and CAL33, low in HNO41 and CAL27 and high in HEp-2 cell lines. Pyrosequencing revealed that the *DUSP2* CpG island was unmethylated in HEp-2 cells and methylated in the other cell lines, with a clear correlation between methylation and *DUSP2* down-regulation (*Figure S1*).

Methylation analysis in primary LA-HNSCC tissues

We performed pyrosequencing analysis of *DUSP2* CpG island methylation in a well-annotated series of 112 cases

Table 2 Distribution of methylated and unmethylated *DUSP2* in tissues at diagnosis (N=112) according to clinopathological characteristics of patients

Characteristic	Methylated (N=51), N (%)	Unmethylated (N=61), N (%)	P value
Gender			0.23
Male (M)	41 (80.4)	54 (88.5)	
Female (F)	10 (19.6)	7 (11.5)	
Primary site			0.195
Oropharynx	8 (15.7)	14 (23.0)	
Hypopharynx	20 (39.2)	21 (34.4)	
Larynx	6 (11.8)	15 (24.6)	
Oral cavity	16 (31.4)	10 (16.4)	
Nasopharynx	1 (2.0)	1 (1.6)	
Performance status			0.98
PS0	22 (43.1)	26 (42.6)	
PS1	27 (52.9)	33 (54.1)	
PS2	2 (3.9)	2 (3.3)	
Tumour size			<0.0001
T1/T2	25 (49.0)	9 (14.8)	
T3/T4	26 (51.0)	52 (85.2)	
NA	0	0	
Nodal status			0.484
N0	3 (5.9)	6 (9.8)	
N1	5 (9.8)	9 (14.8)	
N2/N3	42 (82.4)	46 (75.4)	
NA	1 (2.0)	0	
Grade			0.513
G1/G2	22 (43.1)	33 (54.1)	
G3	27 (52.9)	26 (42.6)	
NA	2 (3.9)	2 (3.3)	
Smoke			0.267
Heavy smokers	45 (88.2)	47 (77.0)	
Non-smokers	2 (3.9)	3 (4.9)	
NA	4 (7.8)	11 (18.0)	
Clinical response			0.32
Complete remission	29 (56.9)	29 (47.5)	
Non-responders	22 (43.1)	32 (52.5)	

DUSP2, dual-specificity-phosphatase-2; NA, not available.

LA-HNSCC from our clinical practice. We excluded patients with obscure cancers (N=3) and HPV16 positive oropharynx tumours (N=14). Clinico-pathological details of the cases are shown in *Tables 2* and *3*. Using 10% mean methylation through methylation variable sites of the amplified fragment of the CpG island as a cut-off, 51/112 (45.5%) of cases were deemed positive for *DUSP2* methylation. Methylation positivity was more frequent among patients with small tumour size (T1/T2) (25/51=49.0% methylated *vs.* 9/61=14.8% unmethylated; P<0.0001), but there was no significant correlation between methylation status and gender, primary tumour site, PS, N, G, smoke and clinical response. Furthermore, there was no association between *DUSP2* methylation and *TP53*

Table 3 Distribution of methylated and unmethylated *DUSP2* in tissues at diagnosis (N=112) according to clinopathological characteristics of patients

Characteristic	*DUSP2* in tissue at diagnosis (N=109)*, N (%)		P value
	Methylated (N=49)	Unmethylated (N=60)	
TP53 mutation			0.292
Wild-type	17 (34.7)	29 (48.4)	
Disruptive	17 (34.7)	14 (23.3)	
Non-Disruptive	15 (30.6)	17 (28.3)	
SNP72 (*TP53*) rs1042522			0.317
Arg/Arg+Arg/Pro	38 (77.6)	51 (85.0)	
Pro/Pro	11 (22.4)	9 (15.0)	

Analysis not performed in 3(*) samples. DUSP2, dual-specificity-phosphatase-2.

mutational status or the rs1042522 polymorphism.

DUSP2 methylation in combination with TP53 status predicts outcome in LA-HNSCC

We asked if methylation of *DUSP2* is a biomarker of clinical outcome in LA-HNSCC treated with CRT, by analysis of time-dependent end points in cases positive or negative for *DUSP2* methylation. There was no difference between cases positive (N=49) and negative for *DUSP2* methylation (N=55) in either OS (P=0.64) nor PFS (P=0.76).

Since *DUSP2* is a transcriptional target for p53, we next asked whether *TP53* and *DUSP2* status together might influence outcomes in the 101 patients for which both parameters were available. We identified 59 mutated patients (58.4%), with 46 missense (78%) and 13 deletions with frameshift effects (22%). In univariate analysis, no difference in OS or PFS was found among patients carrying low (N=22) *vs.* high (N=24) missense mutations *vs.* deletions (N=13), according to EAp53 analysis (29). The same result was obtained when comparing patients carrying disruptive (N=27) *vs.* non-disruptive (N=32) mutations, according to Poeta *et al.* [2007] (28). This allowed us to consider the 59 patients with *TP53* mutation as a unique group (*TP53*-mut) in subsequent analyses. OS was longer in *TP53*-mut *vs.* *TP53*-wt (N=42) cases (P=0.03) (*Figure 1A*). This was not observed in PFS.

Combining *TP53* with *DUSP2* (N=101) and stratifying for *TP53* status, we found that among the 54 unmet-DUSP2 cases, those carrying TP53 mutations (29/54; 53.7%) showed a longer OS (P=0.012), compared to cases with *TP53*-wt (25/54; 46.3%) (P=0.064) (*Figure 1B*). Again, no difference was found in PFS. Instead, in the 47 met-*DUSP2* patients, the stratification for *TP53* didn't show any significant correlation.

DUSP2 methylation status predicts outcome in LA-HNSCC when combined with EGFR

Cases with low EGFR (N=19) had better outcome compared to patients with high-EGFR expression (N=53) although this was not significant (P=0.42). Combining EGFR with *DUSP2* methylation status, we observed that cases with low EGFR and unmethylated *DUSP2* (N=10) had longer OS than those with low-EGFR and met-*DUSP2* (N=9) (P=0.013) (*Figure 2A*). On the contrary, in cases with high EGFR expression (N=53), no difference was seen in OS, although there was a trend suggesting this (*Figure 2B*). There was no difference in PFS in any EGFR/*DUSP2* combination.

Multivariate analysis for DUSP2/EGFR/TP53 combination markers

Finally we tested the utility of the *DUSP2*/EGFR/*TP53* triple combination in predicting clinical outcome in the 70 patients characterised for all those markers. The cumulative P value resulted significant (P=0.005). *DUSP2*-unmet/high-EGFR/TP53-*wt* makeup (N=20) was identified as the major risk factor associated with shorter OS (P=0.0007) (*Figures 3*,*4*).

Instead, patients with *DUSP2*-met/high-EGFR/TP53-*wt*

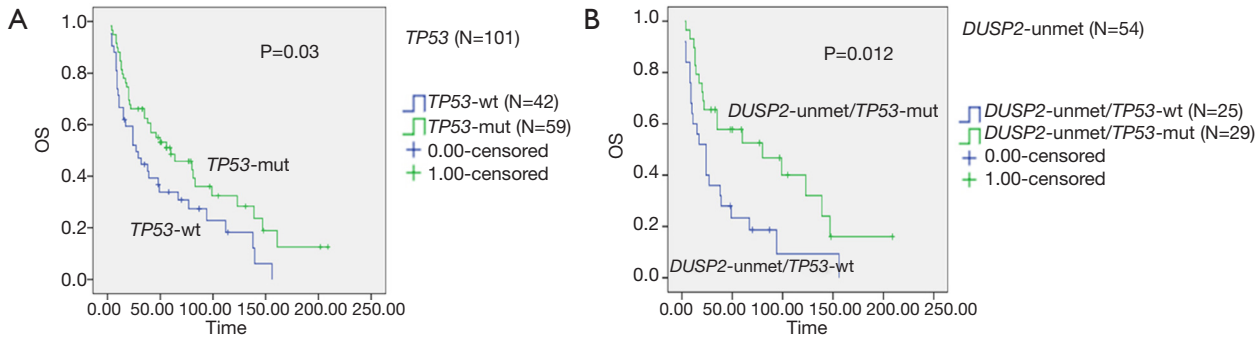

Figure 1 Kaplan-Meier curves for *TP53* status (N=101) and combination for *DUSP2*-unmet/*TP53* status (N=54). (A) LA-HNSCC patients (N=101) carrying *TP53*-mut (N=59) showed a longer OS (months) compared to patients with *TP53*-wt (N=42) (P=0.03, median 60 *vs.* 25.5 months for mut and wt patients, respectively); (B) in LA-HNSCCs with *DUSP2*-unmet tissues (N=54) *TP53*-mut patients (N=29) showed a longer OS (P=0.012), given in months, compared to *TP53*-wt ones (N=25) with a median of 80 and 24 months for mut and wt respectively. LA-HNSCC, locally advanced head and neck squamous cell carcinoma; *DUSP2*, dual-specificity-phosphatase-2.

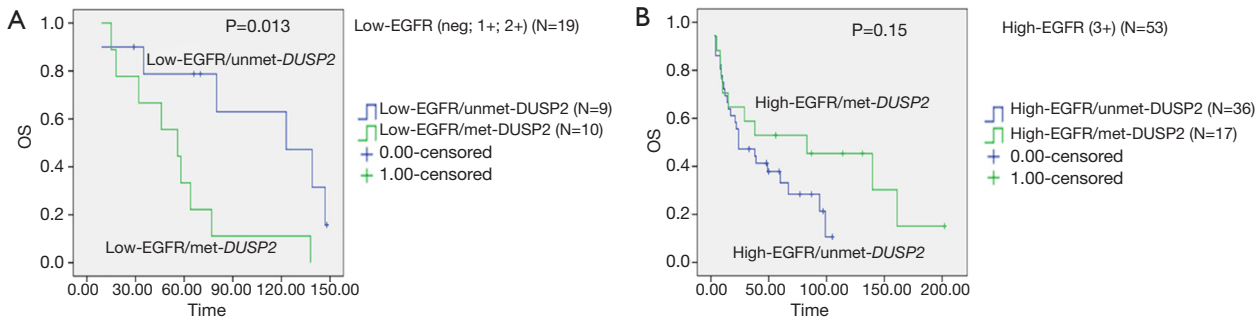

Figure 2 Kaplan-Meier curves for EGFR expression combination with *DUSP2*-unmet (N=19) and *DUSP2*-met (N=53) patients. (A) Among LA-HNSCCs with low-EGFR expression (N=19) *DUSP2*-unmet patients (N=10) showed a better OS (P=0.013), given in months, compared to *DUSP2*-met ones (N=9) with a median of 123 *vs.* 56 for unmet and met tissues, respectively; (B) an opposite trend was seen for LA-HNSCCs with high-EGFR expression (N=53) in which patients with met-*DUSP2* tissues (N=17) showed a numerical longer OS (months) compared with unmet-*DUSP2* ones (N=36) (P=0.15; median 83 *vs.* 24 months for met and unmet, respectively) although this difference was not significant. LA-HNSCC, locally advanced head and neck squamous cell carcinoma; *DUSP2*, dual-specificity-phosphatase-2.

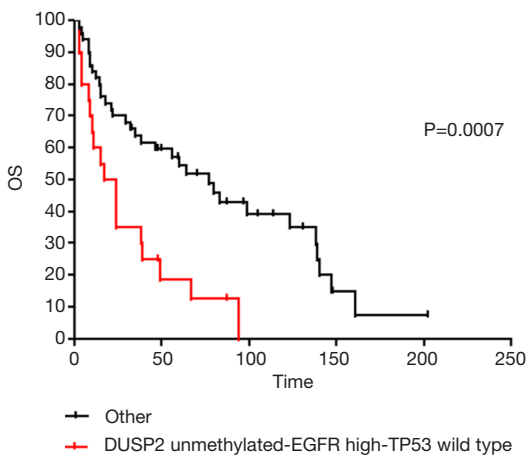

Figure 3 *DUSP2*-unmet/high-EGFR/*TP53-wt* makeup (N=20) is the major risk factor associated with shorter OS (P=0.0007), given in months, identified in our cohort of CRT-treated LA-HNSCC patients. LA-HNSCC, locally advanced head and neck squamous cell carcinoma; *DUSP2*, dual-specificity-phosphatase-2.

A		B = analysis that included variables with P<0.05 in the univariate analysis (signed with *)				C = analysis that included variables with P<0.2 in the univariate analysis (signed with § and *)			
Variable	**P value**	**Variable**	**P value**	**Exp (B)**	**95% CI**	**Variable**	**P value**	**Exp (B)**	**95% CI**
DUSP2 methylation (met *vs.* unmet)	0.5	–	–	–	–	–	–	–	–
EGFR expression (high *vs.* low)	0.44	–	–	–	–	–	–	–	–
Tumour size (T1+T2 *vs.* T3+T4)	0.052*	Tumour size (T1+T2 *vs.* T3+T4)	0.183	0.584	0.264–1.290	Tumour size (T1+T2 *vs.* T3+T4)	0.09	0.485	0.210–1.119
Grading (G1+G2 *vs.* G3)	0.324	–	–	–	–	–	–	–	–
Nodal status (N0+N1 *vs.* N2+N3)	0.39	–	–	–	–	–	–	–	–
Tumor site (oro *vs.* non-o-ro)	0.84	–	–	–	–	–	–	–	–
TP53 status (mut *vs.* wt)	0.037*	TP53 status (mut *vs.* wt)	0.86	0.926	0.396–2.167	TP53 status (mut *vs.* wt)	0.82	1.124	0.412–3.067
*DUSP2*unmet_High-E-GFR_*TP53*-mut	0.402	–	–	–	–	–	–	–	–
*DUSP2*unmet_High-E-GFR_*TP53*-wt	0.001*	DUSP2unmet_High–EGFR_TP53–wt	0.039*	0.387	0.157–0.955	*DUSP2*unmet_High–EGFR_*TP53*–wt	0.28	0.572	0.208–1.572
*DUSP2*unmet_Low-E-GFR_*TP53*-mut	0.111§	–	–	–	–	*DUSP2*unmet_Low–EGFR_*TP53*–mut	0.118	2.326	0.807–6.704
*DUSP2*unmet_Low-E-GFR_*TP53*-wt	0.932	–	–	–	–	–	–	–	–
*DUSP2*met_High-EGFR_*TP53*-mut	0.526	–	–	–	–	–	–	–	–
*DUSP2*met_High-EGFR_*TP53*-wt	0.196§	–	–	–	–	*DUSP2*met_High–EGFR_*TP53*–wt	0.197	2.985	0.566–15.734
*DUSP2*met_Low-EGFR_*TP53*-mut	0.396	–	–	–	–	–	–	–	–
*DUSP2*met_Low-EGFR_*TP53*-wt	0.683	–	–	–	–	–	–	–	–

Omnibus tests of model coefficients (A,B)				**Omnibus tests of model coefficients (A,B)**			
–2 Log likelihood	**Overall (score)**	Change from previous step	Change from previous block	–2 Log likelihood	**Overall (score)**	Change from previous step	Change from previous block
	Chi-square; df; Sig.	Chi-square; df; Sig.	Chi-square; df; Sig.		Chi-square; df; Sig.	Chi-square; df; Sig.	Chi-square; df; Sig.
344.524	12.841; 3; **0.005**	11.485; 3; 0.009	11.485; 3; 0.009	340,052	16.153; 5; **0.006**	15.957; 5; 0.007	15,957; 5; 0.007
A: Beginning block number 0, initial Log likelihood function: –2 Log likelihood: 356,009				A: Beginning block number 0, initial Log likelihood function: –2 Log likelihood: 356,009			
B: Beginning block number 1. Method = enter				B: Beginning block number 1. Method = enter			

Covariate means	
	mean
Tumour size (T1+T2 *vs.* T3+T4)	0.214
TP53 status (mut *vs.* wt)	0.429
*DUSP2*unmet_High-EGFR_*TP53*-wt	0.714

Covariate means	
	mean
Tumour size (T1+T2 *vs.* T3+T4)	0.214
TP53 status (mut *vs.* wt)	0.429
*DUSP2*unmet_High-EGFR_*TP53*-wt	0.714
*DUSP2*unmet_Low-EGFR_*TP53*-mut	0.9
*DUSP2*met_High-EGFR_*TP53*-wt	0.943

Figure 4 Univariate analysis (A) and logistic regression model (B) and (C) in the 70 *DUSP2/EGFR/TP53* patients.

(N=4) showed the longest OS and the highest HR, although not significant, due to their small number (*Figure 4A,B,C*).

Discussion

In the present study we have identified *DUSP2*, a negative regulator of MAP kinases (6), as a novel gene subject to methylation-dependent transcriptional silencing in LA-HNSCC and we show that the quantitative level of *DUSP2* methylation, when combined with EGFR expression and *TP53* mutational status, has utility as a candidate biomarker of clinical outcome in patients treated with CRT. To the best of our knowledge, this is the first demonstration that CpG island methylation regulates *DUSP2* expression in HN cell lines and it is methylated in clinical cases, with 45% of cases in our LA-HNSSC series positive for methylation at diagnosis. Some studies have previously reported the epigenetic inactivation of *DUSP2* in human cancer cell lines (wherein methylation is associated with transcriptional silencing) but not in clinical cases (33).

In vitro, expression of DUSP2 induces apoptosis, inhibits tumour growth and abolishes hypoxia-induced drug resistance (15). These biological properties are all features of tumour suppressor genes and our demonstration of methylation-dependent transcriptional silencing of *DUSP2* in LA-HNSCC affords further experimental evidence in support of this hypothesis. *DUSP2* expression is a determinant of cellular sensitivity to some cytotoxic agents, including cisplatin (15) and targeted therapies such as lapatinib (34). Although *DUSP2* mRNA down-regulation occurs in a number of solid tumours, increased expression levels of *DUSP2* predict a worse OS in serous ovarian carcinoma (35). This apparent contradiction has recently been reported in other potential tumour-suppressor genes (36) including *DUSP1* (7) and *DUSP6* (37).

Low expression levels of *DUSP2* correlates with reduced relapse-free survival in ERBB2-positive breast cancer patients (34), prompting us to examine *DUSP2* methylation in combination with EGFR. Surprisingly, we found that LA-HNSCC cases with *DUSP2* methylation (and therefore likely expressing low levels of *DUSP2*) and high-EGFR had increased OS compared with patients with *DUSP2* unmethylated cases (although this trend did not reach significance), This effect might be associated with a favourable effect of MAPKs that are not inhibited by *DUSP2* {Givant-Horwitz *et al.* [2004]} (35). Instead, low-EGFR patients with *DUSP2*-met tissues showed a shorter OS compared to patients with *DUSP2*-unmet tissues. Our interest in *DUSP2* originated from studies showing that it is also a direct transcriptional target of wild-type p53 and may participate in the *TP53*-dependent DNA damage response to oxidative stress (24). Although some data suggest that *TP53* might be used to predict outcome in HNSCC, its analysis has not become part of the routine evaluation in clinical settings and its role as a prognostic marker remains controversial (38).

In our cohort of LA-HNSCC, *TP53* was the only biomarker, among those analysed, able to independently predict longer OS. Interestingly, we observed longer OS in cases with wild-type *TP53* (58%) compared with mutant *TP53*. This is in apparent contradiction with outcome of CRT as suggested by others (27). Nonetheless, stratifying cases by *DUSP2* methylation status, we observed that those with *DUSP2* methylation and wild-type *TP53* showed a numerical improved OS compared to *TP53* mutant, while, in *DUSP2* unmethylated cases, those with mutant *TP53* showed longer OS.

Mechanistically, it might be speculated that the reduced expression of *DUSP2* induces both *STAT3* and MAPKs activation in tumours with intact *TP53*, while the consequence of normal expression of *DUSP2* in mutated *TP53* cases may increase cellular sensitivity to CRT (39,40).

When we analysed together the markers DUSP2/EGFR/TP53, we found that *DUSP2* silencing at diagnosis was associated with more favourable clinical outcomes when combined with EGFR overexpression and wild-type *TP53*-wt. This highlights the relevance of intact p53 activity for the suppression of HNSCC development.

In conclusion, we show that *DUSP2* methylation might have utility in predicting OS of CRT-treated LA-HNSCC patients suggesting that it may have utility as a biomarker of response to CRT therapies.

DUSP2 function in cancer supports the need for larger clinical studies to further investigate the role of this molecule.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study and the informed consent for biological samples collection and research proposal, obtained from patients, were approved by the Ethical Committee of S. Croce & Carle Teaching Hospital in Cuneo (approval No. 198/13).

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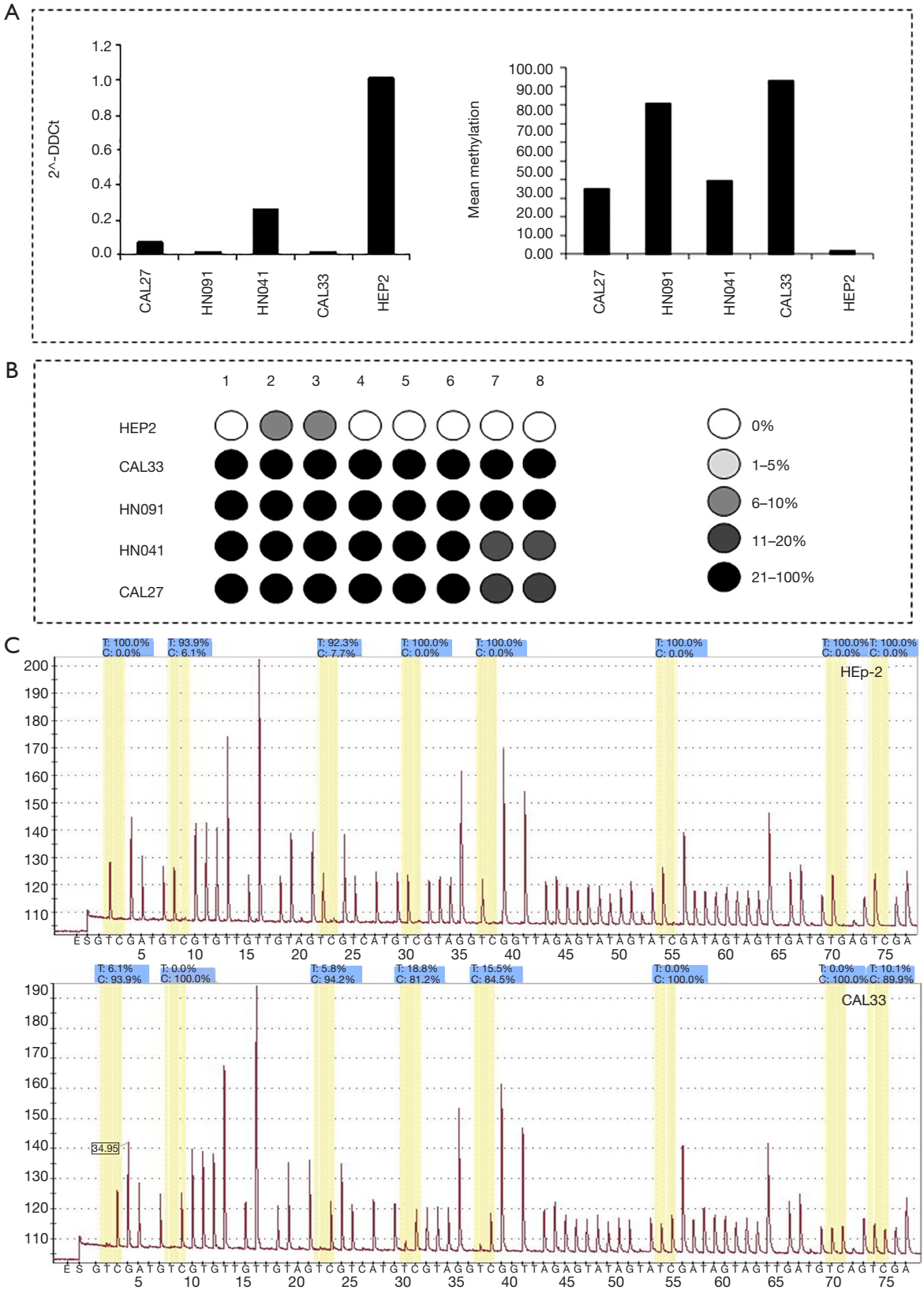

Figure S1 Methylation-dependent transcriptional silencing of DUSP2 in HNSCC cell lines. (A) Expression and methylation analysis of DUSP2 in HNSCC cell lines; (B) DUSP2 methylation profile of HNSCC cell lines. The level of methylation by pyrosequencing is proportional to the degree of shading in the circles, which represent individual CG dinucleotide in the amplified fragment; (C) representative programs showing HEp2 (DUSP2-unmet) and CAL33 (DUSP2-met) cells. DUSP2, dual-specificity-phosphatase-2; HNSCC, head and neck squamous cell carcinoma; CG, Cytosine Guanine.